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# Request for grant of a patent

*(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)*

1. Your reference: P3059 GB PRO

2. Patent application number *(The Patent Office will fill in this part)*: 0205867.5 — 13 MAR 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*:
UNIVERSITY OF NOTTINGHAM
UNIVERSITY PARK
NOTTINGHAM
NG7 2RD

Patents ADP number *(if you know it)*: 79840500 1

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention: POLYMER COMPOSITE LOADED WITH FUNCTIONING MATTER

5. Name of your agent *(if you have one)*: NOVAGRAAF PATENTS LIMITED

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*:
THE CRESCENT
54 BLOSSOM STREET
YORK YO14 1AP

Patents ADP number *(if you know it)*: ~~07296486002~~ 8299166003.

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

| Country | Priority application number *(if you know it)* | Date of filing *(day / month / year)* |
|---|---|---|
| | | |

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

| Number of earlier application | Date of filing *(day / month / year)* |
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8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
*a) any applicant named in part 3 is not an inventor, or*
*b) there is an inventor who is not named as an applicant, or*
*c) any named applicant is a corporate body.*
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YES

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

| | |
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| Continuation sheets of this form | |
| Description | 36 |
| Claim *(s)* | |
| Abstract | |
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10. If you are also filing any of the following, state how many against each item.

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| Priority documents | |
| Translations of priority documents | |
| Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* | |
| Request for preliminary examination and search *(Patents Form 9/77)* | |
| Request for substantive examination *(Patents Form 10/77)* | |
| Any other documents *(please specify)* | |

11. I/We request the grant of a patent on the basis of this application.

Signature Novagraaf Patents Ltd Date

NOVAGRAAF PATENTS LIMITED 12-02-2002

12. Name and daytime telephone number of person to contact in the United Kingdom

PHILIPPA M ALLEN 01904 610586

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# POLYMER COMPOSITE LOADED WITH FUNCTIONING MATTER

The present invention relates to a process for the preparation of a polymer composite comprising contacting polymer with plasticising fluid and functioning matter and isolating loaded polymer, the polymer composite obtained thereby, and apparatus for the preparation thereof, a polymer scaffold, delivery device or the like comprising the composite in suitably sized and shaped form, the use as a pharmaceutical or veterinary product, a human or animal health or growth promoting, structural, fragrance or cosmetic product, an agrochemical or crop protection product, in biomedical, catalytic and like applications, more particularly as a surgical implant, synthetic bone composite, organ module, and the like or for bioremediation, as a biocatalyst or biobarrier and the like.

The use of supercritical fluids in the production of polymers as a plasticising, foaming or purification agent is known. Supercritical fluids (SCFs) act as plasticisers for many polymers, increasing the mobility of the polymer chains. This results in an increase in the free volume within the polymeric material.

Supercritical fluids have found application in incorporation of dyes and other inorganic materials which are insoluble in the supercritical fluid, for example inorganic carbonates and oxides, into polymers with a good dispersion to improve quality, in particular dispersion in products such as paints for spray coating and the like.

Moreover the fluid can be used to foam the polymer by transition to non-critical gaseous state whereby a porous material may be obtained and this has been

disclosed in US 5,340,614, WO91/09079 & US 4,598,006.

SCFs also act as a solvent for impurities (including unreacted monomer and residual conventional solvents) which may be removed during the processing to give high purity products.

SCFs are also of widespread use in cell-breaking for extraction of materials from natural sources such as subcellular matter from mammalian and plant cells and other organic matter, usually involving pulverising the natural source matter i.e. breaking cells in the process of extraction. This is an established and growing technology which is particularly effective for mammalian cells.

Polymers have also been used in biomedical applications to develop materials in which biocompatibility can be influenced to promote favourable tissue responses whilst also producing materials with acceptable mechanical and surface properties. Biofunctional composite materials e.g. calcium hydroxyapatite dispersed in various polymers are well established for orthopaedic, dental and other applications. These materials are prepared with very high loadings of biofunctional inorganic solid, of up to 80%, in the form of a powder, and a composite is formed either by vigorous mixing of the powdered material into the solid or molten polymer, or by polymerisation of the monomers in the presence of suspended inorganic powders. In both cases, the material becomes entrapped within the polymer matrix.

WO 98/51347 (Howdle *et al*) discloses the preparation by SCF processing of biofunctional polymers comprising biofunctional material having the desired mechanical properties both for commercial processing and for implant into a

human or animal host structure such as bone or cartilage, dental and tissue structures into which they are surgically implanted for orthopaedic implant, prosthetic, dental filling or restorative applications, prolonged release applications and the like. Biofunctional material is in particular any pharmaceutical, veterinary, agrochemical, human and animal health and growth promoting, structural, cosmetic and toxin absorbing materials, such as a broad range of inorganic and organic molecules, peptides, proteins, enzymes, oligosaccharides, carbohydrates, nucleic acids and the like.

In the Examples, various materials are introduced, including in a particular example the protein catalase which is processed at 45C under supercritical conditions. Subsequent assay showed retention of enzyme activity. This work is disclosed in more detail in Chem Commun. 2001, 109-110, Howdle et al.

This and other work from the same authors has established that biologically active materials can be mixed with polymers plasticised in SCF in the absence of additional solvent, yet retaining activity of the biological materials.

US 5766637 (Shine *et al*) also discloses introducing biologically active materials into polymers plasticised in supercritical fluid, and gives as sole example the incorporation of a vaccine comprising a virus. The virus is first cooled by refrigeration (ie temperature in excess of 0C), then contacted with SCF for a prolonged period during which temperature is increased to 37C, the polymer is processed, and the virus loaded polymer is then frozen.

Polymers are also being developed in biomedical applications as biological cell-laden scaffolds for use as biomedical inserts such as bone inserts, and as organ

and tissue modules for in vitro and in vivo use, as inserts or for in vivo studies. A considerable effort is being invested in developing scaffolds which encourage growth and development of particular types of living matter and in particular configurations to mimic living systems. Cell-loading is typically by dropping a cellular soup onto the scaffold surface and allowing to permeate in whereby cells are seeded and are found to grow and proliferate into and throughout the scaffold itself on culturing. This is relatively time consuming, the rate limiting factor being the cell growth and proliferation, moreover each scaffold must be configured for the desired growth configuration.

It is an object of the present invention to provide a method for loading cells into polymers directly, i.e. for instant production of cell-laden scaffolds, in one step, in contrast with forming a scaffold and seeding with cells. To date no one has found a means to achieve this, and keeping the cells alive.

"Bacterial inactivation by using near- and supercritical carbon dioxide" Dillow et al, Proc. Natl. Acad. Sci. USA, Vol 96 pp 10344-10348, Aug 1999 discloses contacting bacterial cells with SC $CO_2$ under supercritical conditions of 25 – 35C, 205 bar in the absence and presence of polymer microspheres. Contact times of 0.1 to 4 hours, typically 30 and 45 minutes were employed . Contacting was reported to achieve bacterial inactivation and thus sterilisation of polymer where present. Sterilisation and virus inactivation kits using SCF are available.

We have now surprisingly found, contrary to the indications of Dillow, that the properties of plasticising fluids in general may be employed in the preparation of polymer composites comprising loaded cells and the like distributed throughout the composite, in manner that loaded populations of cells and the

like remain viable after processing. The inventors have found that cryopreserved, desiccated or otherwise preserved cell pellets can be exposed to carbon dioxide under plasticising conditions without interacting with the fluid. The cells were found to remain viable on thawing or rehydrating after the fluid plasticisation.

This is particularly surprising since cells are highly sensitive to a number of conditions, including elevated temperature, contact with solvents, and lack of nutrients and oxygen. Moreover the established prior art use of super critical fluid in cell-breaking and for sterilisation of materials by inactivating bacterial cells teaches away from the present finding.

Accordingly in the broadest aspect of the invention there is provided a process for the preparation of a polymer composite loaded with functioning matter wherein the process comprises contacting a polymer substrate and an amount of functioning matter with a plasticising fluid under plasticising conditions for a period sufficient to plasticise and swell the polymer and incorporate the functioning matter, and releasing the plasticising fluid to obtain the polymer composite, wherein the functioning matter is chemically or physically preserved against harmful effects associated with the plasticising fluid and is maintained in preserved state throughout contact therewith.

Reference herein to functioning matter is to matter capable of performing or exhibiting specific functioning, and whose functioning is harmed by contact with plasticising fluid. For example SCF dissolves or diffuses into biological cells and like enclosed fluid matter such as liposomes with harmful effect on cell functioning or liposome content functioning. Biological cells comprise

liquid contents and SC CO2 can easily penetrate, permeate and cause damage to these contents.

Chemical or physical preservation according to the invention preferably comprises rendering functioning matter dormant, and therefore is reversible. Preferably preservation comprises rendering functioning matter in plasticising fluid-impenetrable state and/or isolating functioning matter contents which facilitate plasticising fluid damage.

Preferably the functioning matter is physically preserved by cryopreservation, i.e. freezing, more preferably at a temperature in the range –10 to –100°C, most preferably – 20 to – 75°C. Techniques are known in the art, for example the matter may be immersed in liquid nitrogen or dry ice containing solvent such as ethanol, or enveloped in dry ice or convection cooled. Cryopreservation agents are suitably employed.

Alternatively functioning matter is physically preserved by dessicating for example by contact with a dessicant, preferably trehalose. Dessication is determined according to known techniques, by weight loss, to agree with the calculated water content. Preferably desiccation is substantially 100%, for example 70% to 100%. Dessication is at reduced pressure as known in the art. Water which facilitates damage in the presence of plasticising fluid is thereby removed.

By means of preservation the liquid contents of the biological cell, for example, are rendered solid and therefore less likely to be harmed by $CO_2$, and moreover the cell is less likely to be penetrated by $CO_2$.

Matter may conveniently be isolated immediately prior to processing, eg 2 hours prior to processing, or may be isolated and stored for periods of the order of 1 week or more prior to processing. Matter may be provided already in isolated state.

Reference herein to loading is to introducing matter internally in uniform or random distribution, throughout the composite cross-section, otherwise known as 3D distribution or internal distribution. Loading may be in a preliminary amount in the case of matter which is capable of subsequent growth to a desired final loading.

Loading may be of any desired polymer volume selected according to desired product quantities and also processing time, as hereinbelow defined. Suitable polymer volumes may be small eg. 5 or 10g polymer, up to multi kg scale.

Preferably functioning matter is provided in dry or wet particulate or powder form suitable for loading into the polymer, and may be of particle size in the range 1 micron to 1 cm, preferably 50 micron to 1000 micron in case of biological cells and aggregates. Functioning matter may be of uniform or non-uniform particle size, depending on practical constraints and the required loading uniformity and distribution. Particles may be used dry or in suspension in a carrier or other agent, preferably a cryopreservation agent.

The product composite may be assessed for preserved functioning, after contact with plasticising fluid loading into polymer. Preferably viability of cells is assessed by light microscopy, or by using an Alamar Blue assay. The Alamar

Blue assay measures cell proliferation, hence viability, and is a REDOX reaction which produces a fluorescent product upon reduction of the reagent by the cells. Fluoresence therefore correlates directly with cell viability.

Preferably in the case that the functioning matter requires nutrients or the like, for example comprises biological cells, the process includes a further step of maintaining the product composite in culture, or under dormant or suspended state conditions prior to culturing. Maintaining the composite in culture comprises providing all cell nutrients required for the survival of the functioning matter and removing any effluent produced, and more preferably comprises providing all nutrients required for proliferation and growth of the functioning matter and removing effluent produced. Preferably maintaining the composite in culture comprises contacting with a source of oxygen, water, carbohydrate and essential minerals, typically in the form of cell culture media as known in the art. The composite may be immersed in culture or may be drip fed, or otherwise contacted with culture media as known in the art. Other techniques encouraging cell growth and fusion, such as cyclic mechanical straining, applied electric field and the like may be employed.

Suitably maintaining the composite under dormant or suspended state conditions comprises maintaining under the prevailing or different preservation conditions employed heretofor during contact with plasticisation fluid..

The polymer is suitably in the solid phase or is a highly viscous, viscous or non-viscous fluid and may present limited or good mixing characteristics. Solid phase polymer may be particulate, eg in the form of granules, pellets, microspheres, powder, or monolithic eg matrix form. Plasticising conditions

comprise conditions of reduced viscosity to plasticise and swell the polymer. It is known that particulate polymer agglomerates on plasticisation to a larger structure. This may revert to a particulate composite or form a monolithic composite on release of plasticising fluid, as hereinbelow defined.

Reference herein to a plasticising fluid is to a fluid which is able to plasticise polymer in its natural state or in supercritical, near critical, dense-phase or subcritical state. Fluid may be liquid or gaseous, and is preferably selected for a suitable density which is capable of plasticising a given polymer, fluid density may be in the range 0.001 g/ml up to 10 g/ml for example 0.001 g/ml up to 2 g/ml.

Plasticising conditions comprises elevated or ambient temperature, and/or elevated or ambient pressure. Fluid may be selected for effective plasticisation of a given polymer under conditions which are amenable to the functioning matter or alternatively fluid is selected by preferred chemical type and suitable plasticising conditions are chosen for that fluid, preferably selecting a first amenable condition (T) and compensating with second condition (P) to obtain desired density.

Preferably the plasticising conditions comprises a desired temperature less than, equal to or greater than the fluids critical temperature (Tc) in the range –200°C to +500°C, preferably –200°C to 200°C. For most fluids this will be in the range approximately 10 to 15°C, 15 to 25°C, 25 to 30°C, 30 to 35°C, 35 to 45°C or 45 to 55°C, most preferably approximately 28 to 33°C ($CO_2$). Other sub ranges may be envisaged and are within the scope of the invention. Preferably the lowest temperature is employed which is compatible with sufficient lowering

of the polymer Tg to achieve plasticisation. To operate at ambient temperature, the process of the invention may require compensation by increase in pressure.

Throughout contact with plasticising fluid, under conditions which typically comprise ambient temperature or elevated temperature, frozen functioning matter is preferably maintained frozen by conducting the process in a vessel and cooling the vessel.

Preferably the plasticising fluid comprises a desired pressure less than, equal to or greater than the plasticising fluids critical pressure (Pc) from in excess of 1 bar to 10000 bar, preferably 1 to 1000 bar, more preferably 2 to 800 bar, most preferably 5 to 75 bar. For most fluids this will be in the range approximately 30 to 40 bar, 40 to 50 bar, 50 to 60 bar, 60 to 75 bar, most preferably approximately 34 to 75 bar (dense phase or supercritical $CO_2$). Other sub ranges may be envisaged and are within the scope of this invention.

Fluid may be provided at plasticising conditions prior to contacting with polymer and functioning matter or may be brought to plasticising conditions in contact with one or both of polymer and functioning matter.

The process is conducted for a suitable contact time which can be employed without prejudicing functioning of matter. For example it is important that the process is conducted with contact time such that there is little or no thawing of frozen functioning matter. In a particular advantage the process is carried out for very short contact time of plasticising fluid and functioning matter of 2 milliseconds up to 10 minutes, more preferably 20 milliseconds to 5 minutes, more preferably 1 second to 1 minute, more preferably 2 to 30 seconds, most

preferably 2 to 15 seconds. In this case a non-uniform distribution may be acceptable. Contact time of plasticising fluid and polymer may be up to 5 hours.

Preferably the polymer and functioning matter are immersed in or contacted with the plasticising fluid in a batchwise process. The components of the polymer composite may be combined in any desired order, prior to, or during application of plasticising conditions. Contacting may be of the components simultaneously or sequentially, for example first contacting polymer and functioning matter and subsequently introducing plasticising fluid, or preferably first contacting polymer and plasticising fluid and subsequently introducing isolated functioning matter. Preferably contact time of preserved functioning matter and plasticising fluid is critical. Preferably preserved functioning matter is loaded into a cooled chamber. Polymer is loaded into a main chamber and plasticised in contact with plasticising fluid, preserved functioning matter is discharged from cooled to main chamber with rapid contacting and release of plasticising fluid. Contact time of polymer and plasticising fluid may be dictated in part by polymer MW, Tg, and/or mass of polymer, whereby contact is sufficient for plasticisation.

The process may be carried out with or without stirring or blending. Blending and conditions may be selected to assist plasticisation or according to the desired uniformity and distribution of loading. In the case that uniform distribution is required the process preferably comprises blending for prolonged period and/or high intensity. In the case that non-uniform distribution is envisaged, the process may be carried out simply with stirring. Preferably the process is carried out under conditions of stirring to combine the functioning

matter and polymer, for purpose of loading but without need for uniform distribution.

Blending may be by physical mixing, pumping, agitation for example with aeration or fluidising gas flow, lamellar flow or otherwise impregnation or diffusion of plasticising fluid throughout the polymer and functioning matter. Stirring is typically with use of stirrers and impellers, preferably helical impellers such as helical ribbon impellers for enhanced blending and the like.

Blending may be for a period of 1 millisecond to 5 hours and maybe for the duration of contacting with plasticising fluid or otherwise. Preferably blending is for the duration of contacting with plasticising fluid for a period corresponding to the period for fluid contacting as hereinbefore defined.

The process comprises subsequently releasing the plasticising fluid. In the case that plasticising conditions comprises elevated pressure release is under reduced pressure conditions, conducted over a desired depressurisation period, whereby the polymer composite is isolated loaded with functioning matter. Depressurisation may be achieved in situ, by depressurising a pressure vessel in which the process is carried out, whereby a monolithic block of polymer composite is obtained. Alternatively the contents of a pressure vessel in which the process is conducted may be discharged into a second pressure vessel at lower pressure whereby a homogeneous powder of polymer composite as hereinbefore defined is obtained by known means.

Depressurisation period may be selected to foam the polymer if desired, and therefore determines the porosity of composite. Transition is preferably rapid

over a period of from 1 ms to 10 minutes, preferably from 1 second to 3 minutes, more preferably from 1 to 3 seconds for high porosity polymer. Alternatively plasticising fluid may be released in manner to allow fluid diffusion out of the polymer, avoiding foaming, to create a non-porous structure. Typically this is achieved by prolonged gradual release of fluid over a period of in excess of 10 minutes up to 12 hours. Preferably transition is to near ambient pressure i.e. substantially 1 atm (101.325 kPa). Pressure release confers further cooling, maintaining the preserved state of frozen functioning matter.

The process may be carried out in the presence or absence of functioning matter compatible carriers or agents such as preservation agents. In a particular advantage the process may be carried out in the presence of solvent carriers and like which might otherwise damage the functioning matter in non-preserved state. Suitable preservation agents include water, to prevent non intentional dessication of hydrated functioning matter, cryopreservation agents which prevent ice crystal formation such as DMSO, trehalose, dextran and the like.

A plasticising fluid as hereinbefore defined may comprise any fluid which is capable of plasticising a desired polymer. As is known in the art such fluids may be subjected to conditions of elevated temperature and pressure increasing density thereof up to and beyond a critical point at which the equilibrium line between liquid and vapour regions disappears. Supercritical fluids are characterised by properties which are both gas like and liquid like. In particular, the fluid density and solubility properties resemble those of liquids, whilst the viscosity, surface tension and fluid diffusion rate in any medium resemble those of a gas, giving gas like penetration of the medium

Preferred plasticising fluids include carbon dioxide, di-nitrogen oxide, carbon disulphide, aliphatic $C_2$-$_{10}$ hydrocarbons such as ethane, propane, butane, pentane, hexane, ethylene, and halogenated derivatives thereof such as for example carbon tetrafluoride or chloride and carbon monochloride trifluoride, and fluoroform or chloroform, $C_6$-$_{10}$ aromatics such as benzene, toluene and xylene, $C_1$-$_3$ alcohols such as methanol and ethanol, sulphur halides such as sulphur hexafluoride, ammonia, xenon, krypton and the like. Typically these fluids may be brought into plasticising conditions at temperature of between −200°C to + 500°C and pressures of in excess of 1 bar to 10000 bar, as hereinbefore defined. It will be appreciated that the choice of fluid may be made according to its properties, for example diffusion and polymer plasticisation. Preferably the fluid acts as solvent for residual components of a polymer composite as hereinbefore defined but not for polymer or functioning matter as hereinbefore defined. Choice of fluid may also be made with regard to critical conditions which facilitate the commercial preparation of the polymer as hereinbefore defined. Supercritical conditions of some fluids are shown in Table 1.

| Fluid | Critical Temperature / °C | Critical Pressure / bar |
|---|---|---|
| Carbon dioxide | 31.1 | 73.8 |
| Ethane<br>Ethylene | 32.4<br>9.3 | 48.1<br>49.7 |
| Nitrous oxide | 36.6 | 71.4 |
| Xenon | 16.7 | 57.6 |
| Fluoroform $CHF_3$ | 26.3 | 48.0 |

| | | |
|---|---|---|
| Monofluoromethane | 42 | 55.3 |
| Tetrafluoroethane | 55 | 40.6 |
| Sulphur hexafluoride | 45.7 | 37.1 |
| Chlorofluoromethane<br>Chlorotrifluoromethane | 29<br>28.9 | 38.2<br>38.7 |
| Nitrogen | -147 | 33.9 |
| Ammonia<br>Cyclohexane<br>Benzene<br>Toluene<br>Trichlorofluoromethane<br>Propane<br>Propylene<br>Isopropanol<br>p-xylene | 132.5<br>280.3<br>289.0<br>318.6<br>198.1<br>96.7<br>91.9<br>235.2<br>343.1 | 111.3<br>40.2<br>48.3<br>40.6<br>43.5<br>41.9<br>45.6<br>47.0<br>34.7 |

Preferably the plasticising fluid comprises carbon dioxide optionally in admixture with any further fluids as hereinbefore defined or mixed with conventional solvents, so-called "modifiers". $CO_2$ is generally approved by regulatory bodies for medical applications, is chemically inert, leaves no residue and is freely available.

The plasticising fluid may be present in any effective amount with respect to the polymer. Preferably the plasticising fluid is provided at a desired concentration in the reaction vessel to give a desired plasticisation and swelling of polymer. Such range may be from 1% to 200% of the polymer weight, e.g. with

plasticising fluid in sufficient excess to achieve 10% to 40% absorption with respect to polymer weight.

The functioning matter may be present in any effective amount with respect to polymer. Proliferative functioning matter may be provided in the process at a desired starting concentration allowing for survival and post processing growth. Typical values are therefore $1x10^{-12}$ wt% to 99.9 wt%, for example $1x10^{-9}$ wt% to 99.9 wt%, preferably 0.01 to 99.9 wt%, more preferably 0.1 to 99.0 wt%, more preferably greater than 0.5 wt% or 1.0 wt% up to 50 wt%.

Biological functioning matter is typically any nutrient dependent, biological matter which is characterised by replication, division, regeneration, growth, proliferation or the like.

Biological functioning matter is preferably selected from any subcellular, cellular or multicellular matter and aggregates and mixtures thereof. Preferably functioning matter is selected from mammalian, plant and bacterial cells including (subcellular) organelles and aggregates thereof including pancreatic islet or liver spheroids and the like; non cellular matter such as liposomes optionally as carrier of matter such as protein or enzymes which become sensitive to dense phase fluid in presence of liposomic water. Cellular matter is more preferably selected from mammalian and plant prokaryotic and eukaryotic cells and mixtures and aggregates thereof, most preferably mammalian cells selected from fibroblasts, chondrocytes, bone forming cells such as osteoblasts and osteoclasts, bone marrow cells, hepatocytes, cardiomycytes, blood vessel forming cells, neurons, myoblasts, macrophages, microvascular endothelium cells and mixtures thereof and collagen. Biological functioning matter may be

naturally occuring or synthetic, for example cells may be genetically modified or mutated in known manner to incorporate, delete or modify components.

Preferably functioning matter is selected from a component, or precursor, derivative or analogue thereof, of a host structure into which implantation or incorporation is desired and preferably comprises matter intended for growth or repair, shielding, protection, modification or modelling of a human, animal, plant or other living host structure for example the skeleton, organs, dental structure and the like; to combat antagonists; for metabolism of poisons, toxins, waste and the like or for synthesis of useful products by natural processes, for bioremediation, biosynthesis, biocatalysis or the like.

In a particular advantage the process of the invention enables instant production of functioning matter laden scaffolds, for example cell laden scaffolds, in one step, in contrast with current practice of forming a scaffold and seeding with cells over a 24 or 48 hour period. This may have particular advantages in the delivery for example of stem and progenitor cells to patients.

In a further advantage we believe that the processing may confer a degree of sterilisation by the plasticising fluid, whereby it selectively inactivates non preserved matter, such as bacteria present in the atmosphere and the like.

The functioning matter may be in any desired form suited for the intended application, for example in solid, semi-solid such as thixotrope or gel form, semi-fluid or fluid such as paste or liquid form, and may be miscible or immiscible but is insoluble in the polymer and dense phase fluid, eg as a suspension. It may be convenient to adapt the functioning matter form to render

it in preferred form for processing and the intended application. The matter is preferably in the form of solid particles having particle size selected according to the intended application. Preferably particle size is of similar or lesser order to that of the polymer composite particle size, and of any pores, preferably $10^{-9}$m - $10^{-2}$m, for example of the order of nanometers, micrometers, millimetres or centimetres, more preferably 50 micron to 1 centimetre for cells and aggregates.

The polymer composite may additionally comprise any additional biofunctional components as disclosed in WO 98/51347, including but not limited to the following examples typically classed as living matter; (pharmaceutical) drugs and veterinary products; agrochemicals as pest and plant growth control agents; human and animal health products; human and animal growth promoting, structural, or cosmetic products including products intended for growth or repair or modelling of the skeleton, organs, dental structure and the like; absorbent biofunctional materials for poisons, toxins and the like.

Pharmaceuticals and veterinary products, i.e. drugs, may be defined as any pharmacologically active compounds that alter physiological processes with the aim of treating, preventing, curing, mitigating or diagnosing a disease.

Drugs may be composed of inorganic or organic molecules, peptides, proteins, enzymes, oligosaccharides, carbohydrates, nucleic acids and the like.

Drugs may include but not be limited to compounds acting to treat the following:

Infections such as antiviral drugs, antibacterial drugs, antifungal drugs, antiprotozal drugs, anthelmintics,

Cardiovascular system such as positive inotropic drugs, diuretics, anti-arrhythmic drugs, beta-adrenoceptor blocking drugs, calcium channel blockers, sympathomimetics, anticoagulants, antiplatelet drugs, fibrinolytic drugs, lipid-lowering drugs;

Gastro-intestinal system agents such as antacids, antispasmodics, ulcer-healing, drugs, anti-diarrhoeal drugs, laxatives, central nervous system, hypnotics and anxiolytics, antipsychotics, antidepressants, central nervous system stimulants, appetite suppressants, drugs used to treat nausea and vomiting, analgesics, antiepileptics, drugs used in parkinsonism, drugs used in substance dependence;

Malignant disease and immunosuppresion agents such as cytotoxic drugs, immune response modulators, sex hormones and antagonists of malignant diseases;

Respiratory system agents such as bronchodilators, corticosteroids, cromoglycate and related therapy, antihistamines, respiratory stimulants, pulmonary surfactants, systemic nasal decongestants;

Musculoskeletal and joint diseases agents such as drugs used in rheumatic diseases, drugs used in neuromuscular disorders; and

Immunological products and vaccines.

Agrochemicals and crop protection products may be defined as any pest or plant growth control agents, plant disease control agents, soil improvement agents and the like. For example pest growth control agents include insecticides, miticides, rodenticides, molluscicides, slugicides, vermicides (nematodes, anthelmintics), soil fumigants, pest repellants and attractants such as pheromones etc, chemical warfare agents, and biological control agents such as microorganisms, predators and natural products;

plant growth control agents include herbicides, weedicides, defoliants, dessicants, fruit drop and set controllers, rooting compounds, sprouting inhibitors, growth stimulants and retardants, moss and lichen controllers and plant genetic controllers or agents;

plant disease control agents include fungicides, viricides, timber preservatives and bactericides; and

soil improvement agents include fertilisers, trace metal additives, bacterial action control stimulants and soil consolidation agents.

Human and animal health or growth promoting products may be defined as any of the above intended for general health purpose, including vitamins, nutrients, steroids, and the like.

Preferred biofunctional components include growth promoters, biocompatibilisers, vitamins, proteins, glycoproteins, enzymes, nucleic acid, carbohydrates, minerals, nutrients, steroids, ceramics and the like; and may include living matter such as spores, viruses, bacteria and the like. In particular growth factors such as basic Fibroblastic Growth Factor, acid Fibroblastic Growth Factor, Epidermal Growth Factor, Human Growth Factor, Insulin Like

Growth Factor, Platelet Derived Growth Factor, Nerve Growth Factor and Transforming Growth Factor; antitumorals such as BCNU or 1, 3-bis (2-chloroethyl) -1-nitrosourea, daunorubicin, doxorubicin, epirubicin, idarubicin, 4-demethoxydaunorubicin 3'-desamine-3' – (3-cyano-4-morpholinyl) – doxorubicin, 4-demethoxydaunorubicin-3' –desamine-3' – (2-methoxy-4-morpholinyl) -doxorubicin, etoposide and teniposide; hormones such as LHRH and LHRH analogues; and steroideals for birth control and/or antitumoral action such as medroxyprogesterone acetate or megestrol acetate, tricalcium phosphate or the class of apatite derivatives, for example calcium hydroxyapatite which functions as a bone or dental component and promotes biocompatability, silicon which functions as a tissue modelling component, and analogues, precursors or functioning derivatives thereof, bioactive species such as collagen, bioglasses and bioceramics, other minerals, hyaluran, polyethyleneoxide, CMC (carboxymethylcellulose), proteins, organic polymers, and the like and components adapted for incorporation as implants into meniscus, cartilage, tissue and the like and preferably promote growth, modelling, enhancing or reinforcing of collagen, fibroblasts and other natural components of these host structures.

Additional biofunctional component(s) may be mixed with the polymer and functioning matter or may be introduced by subsequent soaking or impregnation of functioning matter laden product composite.

Biofunctional components may be present in any desired amount for example as hereinbefore defined for functioning matter. For example a composite may comprise 80 wt% hydroxyapatite, 10 wt% cells, less than 1 wt% growth factor and more than 1 wt% antibiotic.

Accordingly in a preferred embodiment there is provided according to the present invention a process as hereinbefore defined for the preparation of a polymer composite comprising biofunctional matter as hereinbefore defined and loaded with functioning matter as hereinbefore defined wherein the process comprises in a first stage contacting the biofunctional material and polymer and a plasticising fluid as hereinbefore defined under plasticising conditions as hereinbefore defined for a period sufficient to plasticise and swell the polymer and incorporate the biofunctional material and subsequently introducing an amount of functioning matter for a period sufficient to combine with polymer, and releasing the plasticising fluid to obtain the polymer composite, wherein the functioning matter is chemically or physically preserved from harmful effects associated with the plasticising fluid as hereinbefore defined and is maintained in preserved state throughout contact therewith.

If porous, a composite may comprise open or closed cell pores. Composite obtained with a very open porous structure, known as microcellular, is ideal for biomedical and biocatalytic applications for example supporting growth of blood vessels and collagen fibres throughout the matrix, and forming structures resembling bone, meniscus, cartilage, tissue and the like, and providing a structure for throughput of substrate for biocatalysis and bioremediation and the like.

Non-porous, open or closed cell composite may be useful for biodegradable staged or prolonged release delivery applications of functioning matter or biofunctional material not requiring leaching in or out or other access. Release may be in vitro or in vivo and by parenteral, oral, intravenous, application or

surgical for release proximal to the treatment locus, eg in tissue tumor treatment, or hyperthermic bone tumor treatment.

A porous polymer composite may be obtained with uniform or varied porosity, preferably provides pores of at least two different orders of magnitude, for example of micro and macro type, each present in an amount of between 1 and 99% of the total void fraction of the polymer composite.

Reference herein to micro and macro pores is therefore to be understood to be respectively pores of any unit dimension and its corresponding $10^n$ multiple. For example micro pores may be of the order of $10^{-(10-7)}$m with respective macro pores of the order of $10^{-(7-5)}$m, preferably $10^{-(8-7)}$m and $10^{-(6-5)}$m respectively, more preferably of micron and $10^2$ micron order. The pores may be of any desired configuration. Preferably the pores form a network of tortuous interlinking channels, more preferably wherein the micro pores interlink between the macro pores.

Functioning matter may be distributed throughout relatively smaller and relatively larger pores or confined to larger pores. Functioning matter may be embedded in the walls of pores or may be freely supported but not encased in polymer matrix.

Macro pores are suitably of magnitude and distribution whereby embedding with functioning matter to a desired thickness creates release loci into which functioning matter may proliferate and configure in naturally occurring manner. Micropores create a channel structure throughout the polymer composite, which provides an open cell structure for supply and removal of materials, in particular

nutrient and effluent, and for concomitant proliferation and spread of cells throughout the polymer. Different particle size functioning matter may selectively distribute between smaller and larger pores. Other directing means may be employed to direct anisotropic cells (eg nerve, muscle, blood vessel cells) to channels formed by smaller pores, and direct isotropic cells (eg tissue, bone, cornea, marrow) to larger pores.

The process may be controlled in manner to determine the dimensions and void fraction of micro and macro pores and the morphology of the final product. The period for dense phase fluid release determines in part the level of porosity. Additionally the difference in pressure is proportional to porosity. Also a higher critical temperature confers a higher porosity. The composite is suitably obtained with porosity of 15% to 75% or greater, preferably 50% up to 97%.

Suitably the polymer retains its solid or highly viscous fluid form subsequent to release of plasticising fluid, in order to retain the porous structure induced by the fluid.

Further processing of the polymer, for example additional extraction with super critical fluid as known in the art or with other extractants, post-polymerisation and cross-linking, may be subsequently performed as required and as known in the art.

The polymer may be selected from any known polymer, (block) copolymer, mixtures and blends thereof which may be crosslinked or otherwise which is suited for introduction into or association with the human or animal body, plants or other living matter, or in vitro, or for use in the environment in non-toxic

manner. Suitable polymer materials are selected from synthetic biodegradable polymers as disclosed in "Polymeric Biomaterials" ed. Severian Dumitriu, ISBN 0-8247-8969-5, Publ. Marcel Dekker, New York, USA, 1994, bioresorbable polymers synthetic non-biodegradable polymers; and natural polymers. Preferably the polymer is selected from homopolymers, block and random copolymers, polymeric blends and composites of monomers which may be straight chain, (hyper) branched or cross-linked.

Polymer may be of any molecular weight for the desired application, and is suitably in the range of from 1 to 1,000,000 repeat units. Higher molecular weight may be useful for longer release patterns or slower degradation.

Polymers may include but are not limited to the following which are given as illustration only.

Synthetic biodegradable polymers may be selected from:

Polyesters including poly(lactic acid), poly(glycolic acid), copolymers of lactic and glycolic acid, copolymers of lactic and glycolic acid with poly(ethylene glycol), poly(e-caprolactone), poly(3-hydroxybutyrate), poly(p-dioxanone), poly(propylene fumarate);

Preferably polylactides include DD, DL, LL enantiomers and are prepared from D and L lactic acid and glycolic acid monomers, or a combination thereof, or monomers such as 3-propiolactone tetramethylglycolide, b-butyrolactone, 4-butyrolactone, pivavolactone and intermolecular cyclic esters of alpha-hydroxy

butyric acid, alpha-hydroxyisobutyric acid, alpha-hydroxyvaleric acid, alpha-hydroxyisovaleric acid, alpha-hydroxycaproic acid, alpha-hydroxy- alpha-ethylbutyric acid, alpha-hydroxyisocaproic acid, alpha-hydroxy-3-methylvaleric acid, alpha-hydroxyheptanoic acid, alpha-hydroxyoctanoic acid, alpha-hydroxydecanoic acid, alpha-hydroxymyristic acid, alpha-hydroxystearic acid, and alpha-hydroxylignoceric acid. It is most preferred to use lactic acid as sole monomer or lactic acid as the principal monomer with glycolic acid as the comonomer. The latter are termed poly(lactide-co-glycolide) copolymers; particularly suitable are polymers prepared from lactic acid alone, glycolic acid alone, or lactic acid and glycolic acid wherein the glycolic acid is present as a comonomer in a molar ratio of 100:0 to 40:60;

Poly (ortho esters) including Polyol/diketene` acetals addition polymers as described by Heller in: ACS Symposium Series 567, 292-305, 1994;

Polyanhydrides including poly(sebacic anhydride) (PSA), poly(carboxybisbarboxyphenoxyphenoxyhexane) (PCPP), poly[bis(p-carboxyphenoxy) methane] (PCPM), copolymers of SA, CPP and CPM, as described by Tamada and Langer in Journal of Biomaterials Science- Polymer Edition, 3, 315-353,1992 and by Domb in Chapter 8 of the Handbook of Biodegradable Polymers, ed. Domb A.J. and Wiseman R.M., Harwood Academic Publishers;

Poly(amino acids); polyacetals; polyketals; polyorthoesters;

Poly(pseudo amino acids) including those described by James and Kohn in pages 389-403 of Controlled Drug Delivery Challenges and Strategies, American Chemical Society, Washington DC.;

Polyphosphazenes including derivatives of poly[(dichloro) phosphazene], poly[(organo) phosphazenes], polymers described by Schacht in Biotechnology and Bioengineering, 52, 102-108, 1996; and

Azo polymers
Including those described by Lloyd in International Journal of Pharmaceutics, 106, 255-260, 1994.

Synthetic Non-biodegradable Polymers may be selected from:

Vinyl polymers including polyethylene, poly(ethylene-co-vinyl acetate), polypropylene, poly(vinyl chloride), poly(vinyl acetate), poly(vinyl alcohol) and copolymers of vinyl alcohol and vinyl acetate, poly(acrylic acid) poly(methacrylic acid), polyacrylamides, polymethacrylamides, polyacrylates, Poly(ethylene glycol), Poly(dimethyl siloxane), Polyurethanes, Polycarbonates, Polystyrene and derivatives.

Natural Polymers may be selected from carbohydrates, polypeptides and proteins including:

Starch, Cellulose and derivatives including ethylcellulose, methylcellulose, ethylhydroxyethylcellulose, sodium carboxymethylcellulose; Collagen; Gelatin; Dextran and derivatives; Alginates; Chitin; and Chitosan;

Preferably a non biodegradable polymer is selected from polymers such as ester urethanes or epoxy, bis-maleimides, methacrylates such as methyl or glycidyl methacrylate, tri-methylene carbonate, di-methylene tri-methylene carbonate; biodegradable synthetic polymers such as glycolic acid, glycolide, lactic acid, lactide, p-dioxanone, dioxepanone, alkylene oxalates and caprolactones such as gamma-caprolactone.

Polymer substrate may be obtained from its precursors in the process of the invention. The precursors may react to form the polymer substrate(s) in situ during or subsequent to dense phase fluid processing.

The polymer may comprise any additional polymeric components having performance enhancing or controlling effect, for example determining the degree and nature of cross-linking for improved permeability by bodily fluids or pharmaceutically effective agent, flexural and general mechanical properties, electrical properties and the like.

Additional components which may be incorporated during the manufacture of the polymer composite, for example initiators, accelerators, hardeners, stabilisers, antioxidants, adhesion promoters, fillers and the like may be incorporated within the polymer.

If it is desired to introduce an adhesion promoter into the polymer composite, the promoter may be used to impregnate or coat particles of functioning matter prior to introduction into the polymer composite, by means of simple mixing, spraying or other known coating steps, in the presence or absence of fluid as

hereinbefore defined. Preferably coating is performed in conjunction with mixing with fluid as hereinbefore defined whereby excellent coating is obtained. For example the adhesion promoter is dissolved in fluid as hereinbefore defined and the solution is contacted with functioning matter particles as hereinbefore defined. Alternatively the adhesion promoter is introduced into the autoclave during the mixing and/or polymerisation step whereby it attaches to the functioning matter particles in desired manner.

Preferably the total amount of fillers including the functioning matter lies in the region of 0.01-99.9 wt %, preferably 0.1-99 wt%.

In a further aspect of the invention there is provided a polymer composite obtained with the process of the invention as hereinbefore defined.

In a further aspect of the invention there is provided a polymer composite comprising polymer loaded with functioning matter as hereinbefore defined which is non-established. Reference herein to non-established matter is to instantly loaded functioning matter, and to established matter is to loaded and proliferated, grown, adhered or otherwise modified functioning matter. In the case that matter has an absolute need for nutrients, the composite is rendered in suspended state pending supply or is cultured in contact with a nutrient supply with optional effluent removal in manner to enable establishment of functioning matter.

Established cellular matter is suitably present as plaques, rods or other extended structure types, in particular for cellular matter, with cell fusion. Cellular matter may diversify after culturing to provide different cell types.

The composite of the invention may be distinguished from prior art composite prepared by simple impregnation techniques which either comprise functioning matter at the surface only, or which comprise internally proliferated matter, which is established and can be distinguished in form from the non-established loaded matter of the invention.

The polymer composite may be in desired form suitable for the hereinbefore mentioned applications. Suitably the composite may be obtained in granular or monolith form and is preferably in monolith form for use as a scaffold or drug delivery device.

For use as bioremediation, biocatalyst or biobarrier for human or animal or plant matter, the composite may be in a suitable shaped form or may be impregnated into a shaped product, to provide a barrier film, membrane, layer, clothing or sheet.

For use as a structural component, for example comprising the polymer and optional additional synthetic or natural metal, plastic, carbon or glass fibre mesh, scrim, rod or like reinforcing for medical or surgical insertion, the composite may be adapted for dry or wet insertion into a desired host structure, for example may be in powder, pellet, granule or monolith form suited for insertion as a solid monolith into bone or tissue, as fillers or cements for wet insertion into bone or teeth or as solid aggregates or monoliths for orthopaedic implants such as pins, or dental implants such as crowns etc. Insertion may be by injection, surgical insertion and the like.

The polymer composite may be of any desired particle size in the range of 0.1 or 1 micron powders, preferably from 50 micron or 200 micron for cellular loading up to monoliths of the order of 20 centimetres magnitude. It is a particular advantage of the present invention that the polymer composite is obtained in the desired form in uniform size particles such as powder, pellets and the like. Accordingly if it is desired to obtain a random or discrete distribution of particle size the polymer composite may be milled or may be blended from different size batches.

Composite particle size may be controlled according to known techniques by controlled removal of plasticising fluid. If it is desired to obtain particulate composite, the process mixture is suitably removed from the mixing chamber under plasticising conditions into a separate container under ambient conditions through a nozzle or like orifice of desired aperture, and under desired difference of conditions and removal rate, adapted to provide the desired particle size. Spray drying apparatus and techniques may commonly be employed for the technique.

If it is desired to obtain a polymer composite in the form of monoliths, the plasticising fluid is suitably removed using known techniques for foaming polymers. Accordingly the polymer mix is retained in the reaction vessel and conditions are changed from plasticising to ambient at a desired rate to cause removal of the fluid from the polymer mix. Depending on the nature of the polymer it is possible to obtain the monolith in porous foamed state, if desired, having interconnecting pores and channels created by the removal of the plasticising fluid, simply by selecting a polymer consistency which is adapted to retain its foamed state.

Monoliths may be formed into desired shape during the processing thereof, for example by removal of plasticising fluid from a mixing vessel, or from a mould internal to mixing vessel, having the desired monolith shape. Alternatively monolith may be removed from the mixing vessel and cut to desired shape or transferred directly into a mould.

In a further aspect of the invention there is provided a scaffold comprising a polymer composite loaded with functioning matter, optionally additionally comprising biofunctional materials, as hereinbefore defined, suitably sized and shaped for a desired application as hereinbefore defined. The scaffold is suitably provided in suspended state as hereinbefore defined, and may have been cultured subsequent to composite preparation. Preferably the scaffold is frozen, and is in non-established state or established state.

A scaffold according to the invention is suitably in the form of a surgical implant, synthetic bone composite, an organ module, etc. or biocatalyst or biobarrier for synthesis or remediation or the like. The scaffold may be biodegradable or otherwise, for biodegradation in the body and in-growth by native cells, or for biodegradation in the environment after completion of bioremediation avoiding in each case the need for subsequent operation to remove the polymer.

In a further aspect of the invention there is provided an apparatus for use in the preparation of a polymer composite as hereinbefore defined. Suitably the apparatus comprises one or more pressure vessels adapted for temperature and pressure elevation and comprising means for mixing the contents. The pressure

vessel may include means for depressurisation or for discharging of contents into a second pressure vessel at lower pressure. The apparatus comprises means for introduction of functioning matter, dense phase fluid and polymer whilst the vessel is pressurised, as commonly known in the art. Preferably the apparatus comprises a first chamber having cooling means for functioning matter, and a main pressure vessel for contacting dense phase fluid, functioning matter and dense phase fluid and polymer and means to discharge matter from first to main chamber and optionally from main to a second pressure vessel. Preferably discharging means are high velocity and comprising a pressure ram or the like.

The invention is now illustrated in non limiting manner with reference to the following examples and Figures wherein

Figure 1 A – D shows scanning electron micrograph images of composites fabricated by the process of WO 98/51347 (Howdle et al) employed in the present invention; in Images A and B of an internal fracture surface of a monolith composite of calcium hydroxyapatite (40 wt%) and PLGA (60 wt%), at low magnification the distribution of calcium hydroxyapatite throughout the matrix and the production of pores is evident, at higher magnification the intimate mixing of guest particles and polymer is observed; in image C catalase (50% wt) is shown incorporated into a PLGA matrix (50%), micron scale pores in the polymer and the distinctive protein particle morphology are evident; in image D a high surface area microparticle composite (fluorescein (sodium salt) (8 wt%) and polycaprolactone (92 wt%)) are observed produced by direct atomisation, ie after fast depressurisation through an orifice.

Figures 2 and 3 show scanning electron micrograph images and corresponding

mercury porosimetry data for PLA composites fabricated by the process of WO 98/51347 (Howdle et al) employed in the present invention with control of PLA pore structure by changing de-pressurisation conditions; in Figure 2 the image shows presence of a small population of large pores obtained by de-pressurisation over a 2-hour period ("slow"); in Figure 3 the image shows an increase in porosity and a more heterogeneous distribution obtained by de-pressurisation over a 2-minute period ("fast"); data obtained by mercury porosimetry demonstrate that fine control over micropore distribution is achieved by changing only the de-pressurisation rate, with "slow" depressurisation creating pores in the 50 to 500 nm range, whilst "fast" depressurisation is strikingly different and creates pores in the 500 nm to 5 μm range

Figure 4 shows scanning electron micrograph images of cell-laden polymer composite fabricated according to the invention; viability of murine 3T3 fibroblasts is observed, with post processing incubation from Days 1 to 8, as described in the Examples showing spreading and attachment of cells and plaque formation

Figure 5 shows a scanning electron micrograph image of the cell-laden polymer composite of Figure 4 at Day 8 in greater magnification.

Example 1 – Isolation of Functioning matter

Biological cells were prepared as either frozen or dessicated particles. A suspension of mouse 3T3 fibroblasts in 10% DMSO in FCS (foetal calf serum) containing 0.1M HEPES buffer pH 7 was frozen in a mould at –70°C for at least 2 hours (and up to one week) prior to exposure to dense phase $CO_2$. The

resulting cell pellet was immersed in liquid nitrogen and then physically disrupted with a pestle and mortar to form particles with average diameters of between 50 and 1000 micron. DMSO acts as a cryopreservation agent allowing freezing and thawing without damage, as known in the art.

Example 2 – Composite formation of Polymer with homogeneously dispersed isolated Functioning matter, by dense phase fluid processing within the autoclave.

Starting materials were poly (DL-lactic acid) (100 – 200 mg) and frozen mammalian cells obtained from Example 1. The frozen cells were seeded between two part-formed PLA slices and these were placed within an autoclave. The temperature was maintained at 4°C. Carbon dioxide was pumped into the autoclave until a pressure of 1000 psi, 68.03 bar was achieved. A stirrer within the autoclave was rotated to combine the polymer and cell particles. In this case the stirrer was a helical impeller. After 10 seconds of exposure the pressure was rapidly released by opening a valve. The composite comprising polymer loaded with frozen cells was then removed from the autoclave and stored frozen in liquid nitrogen.

Examples 3 and 4

The method of Example 2 was repeated in each case with the autoclave maintained at 20°C and 37°C respectively for shorter contact times of 5 and 1 seconds.

Example 5 - Viability testing

The composites from Examples 2 to 4 were allowed to thaw at room temperature, placed in cell culture media and incubated at 37°C with a 96% air/5% carbon dioxide atmosphere for 8 days. The composites are shown as scanning electron micrograph images at a fracture surface in Figure 3 A – F. Viability of the cells was assessed by confocal microscopy and SEM at Days 1, 4 and 8.

Cell survival was observed using an Alamar Blue assay, fluoresence correlating directly with cell viability. Cells were in a state for proliferation, as seen by the presence of confluent plaques after 8D.

Further aspects and advantages of the invention will be apparent from the foregoing.

Figure 1

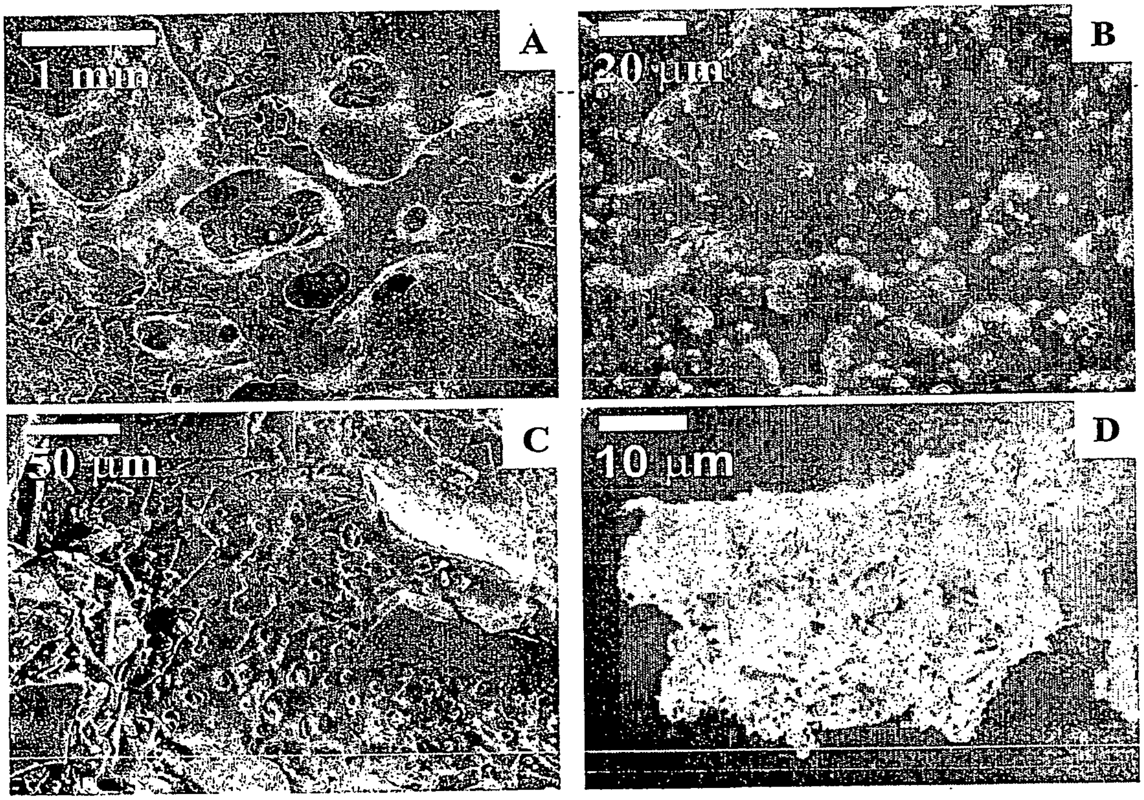
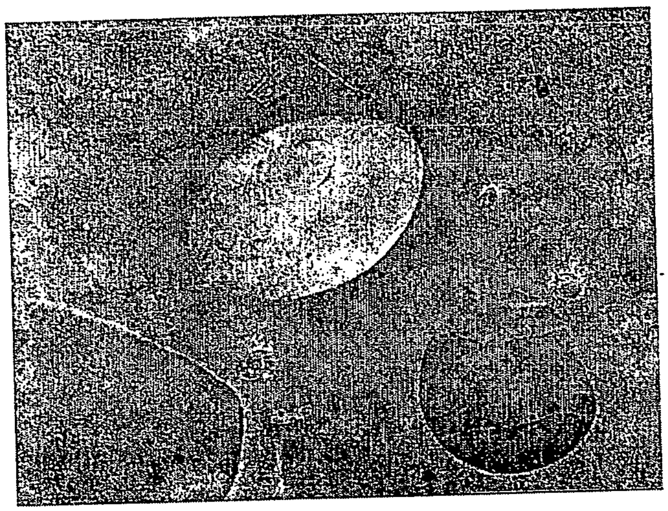
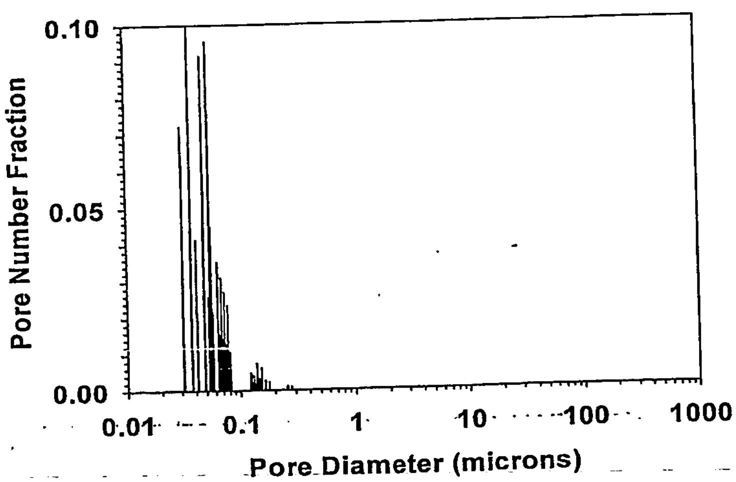
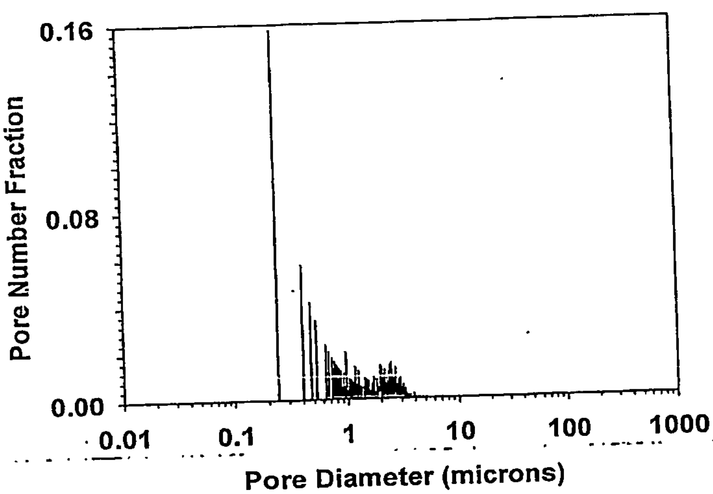


Figure 2



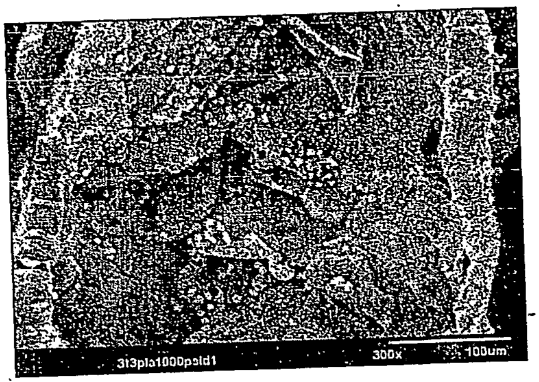

0.10
0.05
0.00
Pore Number Fraction
0.01
0.1
1
10
100
1000
Pore Diameter (microns)

# Figure 3

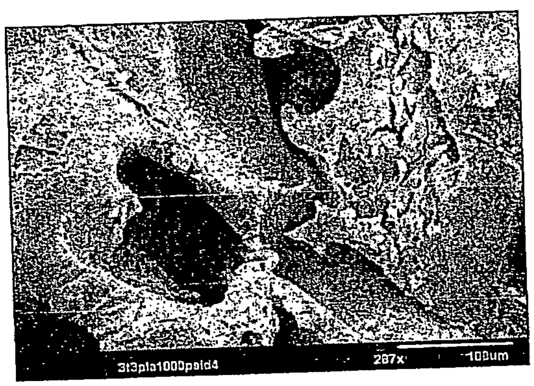

0.16
0.08
0.00
Pore Number Fraction
0.01
0.1
1
10
100
1000
Pore Diameter (microns)

# Figure 4

Day 1




Day 4




Day 8

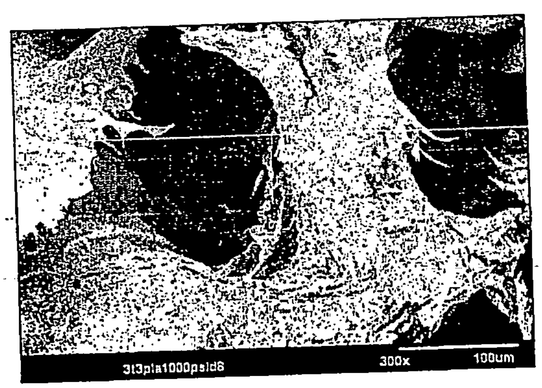
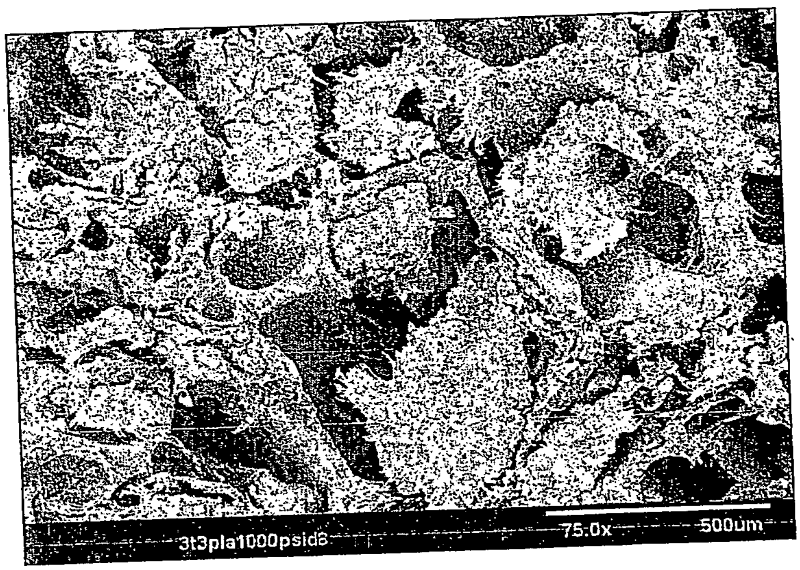


Figure 5

Day 8



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